

Identification of cellular targets of Hepatitis C Virus Non-structural Protein 5A.

Dissertation zur Erlangung des akademischen Grades des
Doktors der Naturwissenschaften (Dr. rer. nat.)

eingereicht im Fachbereich Biologie, Chemie, Pharmazie
der Freien Universität Berlin

vorgelegt von

Tilmann Bürckstümmer
aus Heidelberg

Januar 2005

1. Gutachter: Prof. Dr. Bernd Appel
(Robert-Koch-Institut/ Freie Universität Berlin)

2. Gutachter: Prof. Dr. Volker Erdmann (Freie Universität Berlin)

Disputation am 20. Juni 2005

| | | |
|----------|---|-----------|
| 1 | Introduction | 1 |
| 1.1 | History and classification of HCV..... | 1 |
| 1.2 | Epidemiology and natural history of hepatitis C..... | 1 |
| 1.3 | Immune response..... | 2 |
| 1.4 | Transmission, diagnosis and therapy..... | 5 |
| 1.5 | Genome organization and protein biosynthesis | 6 |
| 1.6 | Viral life cycle..... | 8 |
| 1.7 | Model systems for the analysis of HCV. | 9 |
| 1.8 | Role of NS5A for viral replication..... | 11 |
| 1.9 | NS5A localization and structure. | 12 |
| 1.10 | NS5A modulates host cell signal transduction. | 13 |
| 1.11 | Cellular interaction partners of NS5A..... | 15 |
| 1.12 | Core also modulates host cell signal transduction..... | 17 |
| 1.13 | Liver-pathogenesis in HCV-transgenic mice. | 18 |
| 1.14 | Introduction to c-Raf1 | 19 |
| 1.15 | Activation of c-Raf1 triggers the MAP kinase cascade Raf/MEK/ERK. | 20 |
| 1.16 | MAP kinase-independent functions of c-Raf1 | 22 |
| 1.17 | The role of Raf kinases in cancer..... | 23 |
| 2 | Aim of the study | 25 |
| 2.1 | Identification of novel binding partners of NS5A | 25 |
| 2.2 | Analysis of the interference of NS5A with host cell signal transduction | 25 |
| 2.3 | Establishment of NS5A-transgenic mice as a novel model system for HCV-associated liver pathogenesis..... | 25 |
| 3 | Materials..... | 26 |
| 3.1 | Cell lines..... | 26 |
| 3.2 | Bacterial strains..... | 26 |

| | | |
|----------|--|-----------|
| 3.3 | Antibodies | 26 |
| 3.4 | Kits | 28 |
| 3.5 | Enzymes | 28 |
| 3.6 | Primers..... | 29 |
| 3.7 | Plasmids..... | 30 |
| 3.7.1 | Commercially available plasmids | 30 |
| 3.7.2 | Other plasmids..... | 30 |
| 3.8 | Recombinant baculoviruses | 31 |
| 3.9 | siRNAs..... | 31 |
| 3.10 | Size standards..... | 32 |
| 3.10.1 | DNA standards. | 32 |
| 3.10.2 | Protein standards..... | 32 |
| 3.11 | Reagents for cell culture..... | 32 |
| 3.11.1 | Sf9 and Sf21 cells. | 32 |
| 3.11.2 | HuH-7 cells..... | 32 |
| 3.12 | Mice..... | 32 |
| 3.13 | Radioactive chemicals..... | 33 |
| 3.14 | Chemicals and reagents | 33 |
| 3.15 | Devices | 34 |
| 3.15.1 | Gel electrophoresis | 34 |
| 3.15.2 | Centrifugation..... | 34 |
| 3.15.3 | Liquid chromatography..... | 35 |
| 3.15.4 | Microscopes | 35 |
| 3.15.5 | Other devices..... | 35 |
| 4 | Methods..... | 36 |
| 4.1 | Molecular biology..... | 36 |
| 4.1.1 | Digestion with restriction endonucleases..... | 36 |
| 4.1.2 | Agarose gel electrophoresis..... | 36 |
| 4.1.3 | Determining the concentration of nucleic acids | 37 |
| 4.1.4 | Isolation of DNA fragments from agarose gels..... | 37 |

| | | |
|--------|---|----|
| 4.1.5 | Dephosphorylation of linearized DNA..... | 37 |
| 4.1.6 | DNA ligation..... | 37 |
| 4.1.7 | Polymerase chain reaction (PCR)..... | 38 |
| 4.1.8 | DNA sequencing..... | 39 |
| 4.1.9 | Generation of chemically competent <i>E.coli</i> | 39 |
| 4.1.10 | Transformation of <i>E.coli</i> | 40 |
| 4.1.11 | Plasmid isolation..... | 41 |
| 4.2 | Cell biology..... | 41 |
| 4.2.1 | Cultivation and infection of Sf9 cells..... | 41 |
| 4.2.2 | Cultivation and transfection of HuH-7 cells..... | 41 |
| 4.2.3 | Generation of recombinant baculoviruses..... | 42 |
| 4.2.4 | Plaque assay for recombinant baculoviruses..... | 43 |
| 4.2.5 | Reporter gene assay..... | 43 |
| 4.2.6 | HCV replication assays..... | 44 |
| 4.2.7 | siRNA silencing of c-Raf1..... | 44 |
| 4.3 | Protein biochemistry..... | 45 |
| 4.3.1 | Purification of <i>E.coli</i> -derived NS5A by <i>Strep</i> Tactin Sepharose chromatography..... | 45 |
| 4.3.2 | Purification of Sf9 cell-derived NS5A(211-449) and NS5A by <i>Strep</i> Tactin Sepharose chromatography..... | 45 |
| 4.3.3 | Purification of GST-Raf by glutathione Sepharose chromatography..... | 46 |
| 4.3.4 | Kinase assay..... | 47 |
| 4.3.5 | Affinity chromatography approach to identify potential binding partners of NS5A..... | 47 |
| 4.3.6 | SDS-PAGE..... | 47 |
| 4.3.7 | Silver staining of SDS-polyacrylamide gels..... | 49 |
| 4.3.8 | Western blot..... | 50 |
| 4.4 | Immunological methods..... | 51 |
| 4.4.1 | Generation and purification of the NS5A-specific antiserum..... | 51 |
| 4.4.2 | Immunoprecipitation..... | 52 |
| 4.4.3 | Immunofluorescence analysis..... | 53 |
| 4.5 | Generation and analysis of NS5A-transgenic mice..... | 53 |
| 4.5.1 | Generation of NS5A-transgenic mice..... | 53 |

| | | |
|----------|--|-----------|
| 4.5.2 | Detection of the NS5A-transgene by PCR analysis | 54 |
| 4.5.3 | RNA isolation and RT-PCR analysis..... | 55 |
| 4.5.4 | Analysis of NS5A expression by Western blotting..... | 56 |
| 4.5.5 | Immunohistochemical analysis of liver cryo-sections..... | 56 |
| 5 | Results | 57 |
| 5.1 | Generation and purification of an NS5A-specific antiserum..... | 57 |
| 5.1.1 | Generation of an antiserum directed against the C-terminus of NS5A..... | 57 |
| 5.1.2 | Analysis of the NS5A-specific antiserum..... | 58 |
| 5.1.3 | Purification of the NS5A-specific antiserum..... | 59 |
| 5.2 | Identification of c-Raf1 as a novel binding partner of NS5A..... | 62 |
| 5.2.1 | Purification of NS5A | 62 |
| 5.2.2 | c-Raf1 binds to immobilized NS5A..... | 63 |
| 5.2.3 | NS5A and c-Raf1 can be coimmunoprecipitated from Sf21 cells..... | 64 |
| 5.2.4 | c-Raf1 is colocalized with NS5A in the replication complex..... | 66 |
| 5.2.5 | NS5A interacts with the catalytical domain of c-Raf1..... | 68 |
| 5.3 | MAP kinase signaling and HCV replication..... | 70 |
| 5.3.1 | Initial characterization of HCV replicon cells..... | 70 |
| 5.3.2 | BAY43-9006 inhibits the protein kinase C-induced activation of the MAP kinase cascade..... | 72 |
| 5.3.3 | Inhibition of c-Raf1 by BAY43-9006 negatively affects HCV replication..... | 75 |
| 5.3.4 | BAY43-9006-mediated inhibition is not due to a block in cell proliferation..... | 76 |
| 5.3.5 | Downregulation of c-Raf1 by siRNA leads to a decrease in HCV replication..... | 77 |
| 5.3.6 | Inhibition of MEK by PD98059 positively affects HCV replication..... | 78 |
| 5.4 | NS5A does not alter c-Raf1-mediated signal transduction..... | 81 |
| 5.4.1 | NS5A does not affect ERK phosphorylation..... | 81 |
| 5.4.2 | NS5A does not modulate the activity of SRF, AP-1, NF- κ B or STAT-3..... | 82 |
| 5.4.3 | HCV replicon cells do not differ from naïve HuH-7 cells with regard to SRF-, AP-1-, NF- κ B- or STAT-3-activation..... | 84 |
| 5.5 | Analysis of NS5A phosphorylation..... | 87 |
| 5.5.1 | Purification of GST-Raf derived from Sf9 cells..... | 87 |

| | | |
|----------|--|-----------|
| 5.5.2 | Purified GST-Raf is active whereas GST-Raf K375W is not. | 88 |
| 5.5.3 | GST-Raf is contaminated with kinases that phosphorylate NS5A. | 89 |
| 5.5.4 | GST-Raf purified under highly stringent conditions specifically phosphorylates NS5A. | 91 |
| 5.5.5 | Inhibition of c-Raf1 leads to a loss of NS5A hyperphosphorylation <i>in vivo</i> | 92 |
| 5.6 | Generation and analysis of NS5A-transgenic mice..... | 93 |
| 5.6.1 | Generation of transgenic mice..... | 93 |
| 5.6.2 | NS5A is expressed in the liver of transgenic mice..... | 94 |
| 5.6.3 | NS5A expression is also detected at the protein level. | 95 |
| 6 | Discussion | 99 |
| 6.1 | NS5A purification | 99 |
| 6.2 | Interaction of c-Raf1 with NS5A..... | 99 |
| 6.3 | Localization of NS5A and c-Raf1..... | 101 |
| 6.4 | MAP kinase signaling and HCV replication..... | 101 |
| 6.4.1 | Advantages and drawbacks of small-molecule kinase inhibitors..... | 101 |
| 6.4.2 | Impact of c-Raf1- and MEK-inhibition on HCV replication..... | 102 |
| 6.4.3 | Kinetics of HCV replication and viral gene expression..... | 103 |
| 6.4.4 | HCV replication is coupled to host cell proliferation | 104 |
| 6.4.5 | HCV replicons as model systems for HCV replication | 104 |
| 6.5 | Impact of NS5A on c-Raf1-induced signal transduction..... | 105 |
| 6.5.1 | NS5A does not modulate the MAP kinase cascade c-Raf1/MEK/ERK..... | 105 |
| 6.5.2 | NS5A might alter MAP kinase-independent functions of c-Raf1..... | 106 |
| 6.5.3 | NS5A does not activate NF- κ B signaling | 106 |
| 6.6 | NS5A phosphorylation by c-Raf1..... | 108 |
| 6.6.1 | c-Raf1 is an NS5A kinase | 108 |
| 6.6.2 | NS5A hyperphosphorylation and HCV replication | 110 |
| 6.7 | Kinase inhibitors as therapeutic agents..... | 111 |
| 6.8 | Impact of c-Raf1 on HCV-associated liver pathogenesis | 113 |
| 6.9 | Integrity of the c-Raf1/MEK/ERK pathway affects viruses other than HCV..... | 114 |
| 6.10 | Working model and future perspectives..... | 115 |

| | | |
|----|-----------------------|-----|
| 7 | Summary..... | 117 |
| 8 | Zusammenfassung | 119 |
| 9 | Literature | 121 |
| 10 | Abbreviations | 132 |

7 Summary

Hepatitis C virus (HCV) is a small RNA virus that causes severe liver pathogenesis in humans. It encodes for 3 structural and at least 7 non-structural proteins. The non-structural protein 5A (NS5A) has been implicated in the deregulation of host cell signal transduction, interfering with signaling pathways that regulate cell growth, cell proliferation and cell survival. Modulation of these signaling pathways is of potential interest with regard to viral replication, immune evasion and virus-associated liver pathogenesis. Therefore, this study was focused on the identification of cellular targets of NS5A that might trigger the NS5A-mediated deregulation of host cell signal transduction.

Using an affinity chromatography approach, c-Raf1 was identified as a novel binding partner of NS5A. Binding was confirmed by immunoprecipitation using baculovirus-infected Sf21 cells. NS5A and c-Raf1 were found to colocalize in transiently-transfected HuH-7 cells and HCV replicon cells. Furthermore, deletion mutants of NS5A that are localized in the nucleus cause the nuclear translocation of endogenous c-Raf1. This demonstrates that NS5A and c-Raf1 interact in a physiologically relevant model system, ie. in human hepatoma cells.

Next, it was analyzed how modulation of c-Raf1 activity affects HCV replication. Inhibition of c-Raf1 by the means of a small-molecule inhibitor (BAY43-9006) lead to a decrease in HCV replication. Reducing c-Raf1 expression by the means of siRNA lead to a similar reduction of HCV replication, arguing that integrity of c-Raf1 is required for viral replication. Disruption of the MAP kinase cascade at the level of MEK (U0126) did not affect viral replication. These data suggest that the c-Raf1-mediated reduction of HCV replication is not due to the disruption of the MAP kinase cascade. Furthermore, these data highlight the exclusive role of c-Raf1 in viral replication.

In the following set of experiments, the impact of NS5A on c-Raf1-mediated signal transduction was investigated. Surprisingly, the level of ERK phosphorylation was not affected by the presence of NS5A. Moreover, NS5A did not affect the level of basal activation of SRF, AP-1, NF- κ B or STAT3 – either if expressed as an individual protein or if expressed in the HCV polyprotein context. This argues that NS5A does not modulate c-Raf1-mediated signal transduction.

Since NS5A is a phosphoprotein and c-Raf1 is a kinase, the impact of c-Raf1 on NS5A phosphorylation was analyzed. To that end, both NS5A and GST-Raf were purified by affinity

chromatography. Phosphorylation of NS5A by GST-Raf was reconstituted *in vitro* using highly purified GST-Raf. An inactive mutant of GST-Raf (K375W) did not phosphorylate NS5A, arguing that phosphorylation of NS5A was specific on behalf of c-Raf1. This suggests that c-Raf1 is an NS5A kinase *in vitro*. Moreover, c-Raf1 inhibition by the means of BAY43-9006 lead to a decrease in NS5A hyperphosphorylation in HCV replicon cells, arguing that c-Raf1 contributes to NS5A phosphorylation *in vivo*.

In conclusion, this study describes a novel cellular binding partner of HCV NS5A that is essential for NS5A phosphorylation and HCV replication. Among the multitude of binding partners described for NS5A, there are few binding partners that fit these criteria and provide an impact on understanding this exciting, though enigmatic protein.

8 Zusammenfassung

Das Hepatitis-C Virus (HCV) ist ein kleines RNA-Virus, das beim Menschen schwere Lebererkrankungen hervorruft. Es kodiert für 3 Struktur- und mindestens 7 Nichtstrukturproteine. Das Nichtstrukturprotein 5A (NS5A) moduliert verschiedene Signaltransduktionswege der Wirtszelle, die für die Regulation von Zellwachstum und Zellproliferation von zentraler Bedeutung sind. Die Deregulation dieser Signalwege ist potenziell relevant im Hinblick auf die Virusreplikation, die Immunantwort des Wirts sowie die HCV-assoziierte Leberpathogenese. Aus diesem Grund war das Ziel der vorliegenden Arbeit, zelluläre Faktoren zu identifizieren, die für die NS5A-vermittelte Modulation zellulärer Signalwege verantwortlich sind.

Mit Hilfe eines affinitätschromatographischen Ansatzes wurde c-Raf1 als neuer Bindungspartner von NS5A identifiziert. Die Interaktion wurde in Immunpräzipitationsexperimenten aus Baculovirus-infizierten Sf21-Zellen bestätigt. Des Weiteren wurde beobachtet, dass NS5A und c-Raf1 in transient-transfizierten HuH-7-Zellen sowie in HCV-Replikon-Zellen kolokalisiert sind. N-terminale Deletionsmutanten von NS5A, die im Zellkern lokalisiert sind, bewirken eine Translokation von endogenem c-Raf1 in den Kern. Das zeigt, dass NS5A und c-Raf1 in einem physiologisch-relevanten Modellsystem interagieren.

In der Folge wurde untersucht, wie sich eine Modulation der c-Raf1-Aktivität auf die Virusreplikation auswirkt. Die Hemmung von c-Raf1 mittels eines synthetischen Inhibitors (BAY43-9006) führte zu einer Verminderung der HCV-Replikation. Dasselbe wurde beobachtet, wenn die Expression von c-Raf1 durch siRNAs reduziert wurde. Das legt den Schluss nahe, dass die Integrität von c-Raf1 eine Voraussetzung für die Virusreplikation darstellt. Die Hemmung der MAP-Kinase-Kaskade auf der Ebene von MEK (U0126) hatte keinen Einfluss auf die Virusreplikation. Das bedeutet, dass der Effekt von c-Raf1 auf die Virusreplikation nicht durch eine Hemmung der MAP-Kinase-Kaskade bedingt ist. Im Übrigen unterstreicht dieses Experiment die besondere Bedeutung von c-Raf1 für die Virusreplikation.

Außerdem wurde analysiert, inwieweit NS5A einen Einfluss auf die c-Raf1-vermittelte Signaltransduktion hat. Dabei wurde beobachtet, dass die Phosphorylierung von ERK, einem zentralen Effektor von c-Raf1, in Gegenwart von NS5A unverändert ist. Des Weiteren trägt NS5A nicht zur basalen Aktivierung der Transkriptionsfaktoren SRF, AP-1, NF- κ B oder STAT3 bei. Dies gilt sowohl für NS5A als isoliertes Protein als auch für NS5A im Kontext des HCV-

Polyproteins. Das legt den Schluss nahe, dass NS5A keinen Einfluss auf die c-Raf1-vermittelte Signaltransduktion hat.

Da NS5A ein Phosphoprotein und c-Raf1 eine Kinase ist, war es nahe liegend zu untersuchen, inwieweit NS5A durch c-Raf1 phosphoryliert werden kann. Zu diesem Zweck wurden NS5A und GST-Raf durch Affinitätschromatographie gereinigt. Die gereinigten Proteine wurden in einem Kinase-Assay umgesetzt. Dabei konnte gezeigt werden, dass hoch-reines GST-Raf NS5A phosphoryliert. Eine inaktive Mutante von GST-Raf (K375W) war nicht in der Lage, NS5A zu phosphorylieren. Das bedeutet, dass die Phosphorylierung im Hinblick auf c-Raf1 spezifisch war und lässt den Schluss zu, dass c-Raf1 eine NS5A-Kinase ist. In der Folge wurde untersucht, wie sich eine Inhibition von c-Raf1 (durch BAY43-9006) auf die Hyperphosphorylierung von NS5A auswirkt. Es wurde beobachtet, dass die Inhibition von c-Raf1 den Verlust der Hyperphosphorylierung von NS5A zur Folge hat. Das bedeutet, dass c-Raf1 die NS5A-Phosphorylierung auch *in vivo* beeinflusst.

Zusammenfassend kann gesagt werden, dass in dieser Arbeit ein neuer zellulärer Bindungspartner von NS5A identifiziert wurde, der von zentraler Bedeutung für die Phosphorylierung von NS5A und die Virusreplikation ist. Unter den zahlreichen Bindungspartnern von NS5A gibt es nur wenige, die diese Kriterien erfüllen und die zum Verständnis dieses spannenden und zugleich rätselhaften Proteins beitragen.